Title

Trophoblast lineage-specific differentiation and associated alterations in preeclampsia and fetal growth restriction.

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Abstract

The human placenta is a poorly-understood organ, but one that is critical for proper development and growth of the fetus in-utero. The epithelial cell type that contributes to primary placental functions is called "trophoblast," including two main subtypes, villous and extravillous trophoblast. Cytotrophoblast and syncytiotrophoblast comprise the villous compartment and contribute to gas and nutrient exchange, while extravillous trophoblast invade and remodel the uterine wall and vessels, in order to supply maternal blood to the growing fetus. Abnormal differentiation of trophoblast contributes to placental dysfunction and is associated with complications of pregnancy, including preeclampsia (PE) and fetal growth restriction (FGR). This review describes what is known about the cellular organization of the placenta during both normal development and in the setting of PE/FGR. It also explains known trophoblast lineage-specific markers and pathways regulating their differentiation, and how these are altered in the setting of PE/FGR, focusing on studies which have used human placental tissues. Finally, it also highlights remaining questions and needed resources to advance this field.

Introduction

Trophoblast (derived from the Greek word "tropho," meaning to feed) is the epithelial cell in the placenta, a transient organ which plays a pivotal role in fetal growth and development during pregnancy [1]. Trophoblast are derived from trophectoderm (TE), the cells on the outer part of the blastocyst-stage embryo. In human, the TE expands during the early post-implantation period to form a shell around the embryo, composed mostly of proliferative cytotrophoblast (CTB), the putative trophoblast "stem" cell in the placenta [1,2]. CTB subsequently differentiate down two main lineages: in floating villi, they fuse to form syncytiotrophoblast (STB), which form the main barrier against pathogens while also serving as the gas and nutrient exchange interface, while in anchoring villi, they undergo a modified form of epithelial-to-mesenchymal transition to differentiate into invasive extravillous trophoblast (EVT) [1-3]. The latter are composed of two subtypes of cells, one of which invade deeply into the uterine wall as groups of cells, termed interstitial EVT, and another which remodel and line decidual arterioles as endovascular EVT, allowing for maternal blood flow to be established into the intervillous space [1]. Differentiation into both these lineages is important for development of a fully-functioning placenta, which can support growth of the fetus in utero. Abnormal differentiation and function of these two lineages has been associated with pregnancy disorders, including miscarriage, preterm birth, preeclampsia (PE), and fetal growth restriction (FGR) [4,5]. This review will focus on describing trophoblast subtypes as organized within the normal placenta, and discuss how the organization of these lineages is disturbed in pregnancy complications, with a focus on placental insufficiency associated with PE and FGR. Finally, it will also highlight gaps in knowledge, where more research is needed in understanding the relationship between abnormal trophoblast differentiation and placental function.

Trophoblast lineages and their organization within the placenta

Two commonly-used markers for distinguishing villous (CTB and STB) and extravillous trophoblast (EVT) are EGFR and HLAG, respectively [6]. Early in gestation, the trophoblastic shell surrounding the embryo is composed of primary villi [1-3], whose trophoblast is highlighted by EGFR, as well as multiple layers of $HLAG^+$ EVT, among which are abundant multinucleated giant cells (**Figure 1a-b**). The origin of these giant cells has been debated: on one hand, their location within the EVT layers and increasing numbers within the uterine myometrium with increasing gestation [7] suggest they are part of this lineage. On the other hand, the predominant expression of EGFR in these cells suggests that, at least in early gestation, they are in fact of villous origin, potentially representing STB fragments, entrapped within the EVT shell (**Figure 1c-d**).

Later in gestation, EGFR and HLAG staining continue to represent villous and extravillous lineages, respectively. EGFR marks the trophoblast surrounding the floating villi, but also marks the layer of trophoblast at the chorionic plate (fetal surface), while HLAG primarily highlights EVT at the basal plate (maternal surface), but also marks EVT present just below the chorionic plate and surrounding some villi within intraplacental trophoblast islands (**Figure 2a-c**). Interestingly, while the majority of EVT at the basal plate express HLAG, occasional multinucleated giant cells can be seen that are double-positive for EGFR and HLAG (**Figure 2d**), indicating a potentially different cell-of-origin of these cells in later gestation placenta.

Within the $EGFR^+$ villous trophoblast, CTB stem cells, visualized by TP63 immunoreactivity, are abundant in early gestation, comprising a layer that is adjacent to the

basement membrane (**Figure 3a**) [8]. Little is known about the heterogeneity of this cell layer, although single cell RNAseq analysis is beginning to shed some light on this subject [9-11]. Our own work has pointed to one marker, CDX2, which appears to highlight a subset of these villous CTB (**Figure 3b**). In normal gestation, $CDX2^+$ CTB are present mostly near the chorionic plate and disappear after first trimester [12]. The CTB layer itself starts to become discontinuous in the second trimester; by term, only 1-2 CTB can be found per terminal villus [8]. This is reflected in a decrease in PCNA staining over gestation, as CTB are the proliferative cells within the trophoblastic compartment [13].

While there are likely many more human trophoblast subtypes than described above, few have been described or characterized, beyond mere identification with a single marker, and fewer still have been functionally defined. Nevertheless, in the remainder of this review, we will describe changes in trophoblast differentiation and related regulatory pathways, as revealed by a combination of morphology, lineage-specific marker expression, and functional studies (where available), associated with disorders of placental insufficiency, with a focus on PE and FGR.

Preeclampsia and Fetal Growth Restriction: Clinical Synopsis

Pre-eclampsia (PE) is a common pregnancy disorder that is characterized by new-onset hypertension and proteinuria in the latter half of pregnancy, and affects 5-8% of all pregnancies worldwide [14-16]. The incidence of PE in the United states has been on the rise since the 1990s, and it is currently one of the leading causes of maternal and neonatal morbidity and mortality [14]. PE is a heterogeneous disease, and can manifest itself at different gestational ages, with varying degrees of severity. Recently, a "two-stage" model has been developed to describe the underlying pathophysiology, with the first stage being deficient placentation and second stage characterized by systemic vascular inflammation [17]. PE, particularly severe early-onset PE, presenting before 34 weeks gestational age, can be accompanied by fetal growth restriction (FGR), defined as birthweight less than 10^{th} percentile adjusted for sex and gestational age [18]. However, FGR can also be present in isolation, without a simultaneous maternal syndrome [5, 18,19]. While FGR can also be multifactorial, PE and isolated FGR have in common, both some clinical features, including abnormal umbilical doppler studies, as well as underlying etiologies, including defective placentation and systemic maternal vascular dysfunction [5,19]. Both PE and FGR are often associated with a small placenta at delivery, and show various combinations of villous hypermaturity, infarction, and decidual vasculopathy, on histopathologic examination [1,2]. Recent studies have used a combination of gene expression profiling and histopathology to further separate PE and FGR into unique subtypes, with the potential to identify biomarkers with improved diagnostic accuracy [20-23]. However, trophoblast lineage-specific differentiation has yet to be described in these different disease subtypes. Below, we will describe abnormalities in both villous and extravillous trophoblast associated with PE/FGR.

Abnormalities of villous trophoblast associated with PE/FGR

Normal villous trophoblast development involves regular branching of the villous tree, with fusion of underlying CTB into the overlying syncytium within these structures [2]. The process of fusion is regulated through Glial Cells Missing-1 (GCM1), a transcription factor, which is expressed in a subgroup of CTB, induces cell cycle arrest, and mediates cell-cell fusion through induction of SYNCYTIN genes [24, 25]. Downregulation of GCM1 in denuded floating villi has been shown to promote CTB proliferation and inhibit syncytial regeneration [26]. Syncytialization involves induction of two fusogenic genes, SYNCYTIN 1 and 2, in human

chorionic villi, both of which were acquired from human endogenous defective retroviruses [27,28]. The syncytium undergoes significant turnover through shedding of a combination of extracellular vesicles and microparticles (sometimes referred to as trophoblast "deportation"), which, over time, with decreased fusion of underlying CTB, results in formation of nuclear aggregates, called syncytial knots [29]. The number of syncytial knots increases with increasing gestational age, and is thus a reflection of villous maturity [30]. Syncytial knots are composed primarily of condensed nuclei with transcriptionally inactive heterochromatin [31]. The presence of syncytial knots in normal term placentas is also associated with a small spike in TUNEL staining, a further reflection of placental aging [13].

PE/FGR placentas often show poor villus branching, described as "distal villous hypoplasia," accompanied by increased numbers of syncytial knots adjusted for gestational age, consistent with "accelerated villous maturation" [32]. The latter changes were originally described by Tenney and Parker in 1940, as degenerative changes associated with "toxemia of pregnancy," and hence are often referred to in the literature as "Tenney-Parker changes" [33]. These changes are thought to arise due to more turbulent flow of maternal blood into the intervillous space of PE/FGR placentae, resulting in increased oxidative stress [34]; in fact, syncytial knots are positive for markers of oxidative damage, such as 8-oxo-deoxyguanosine [31]. The syncytial knots are also at least one source of circulating soluble VEGF receptor-1 (sVEGFR-1, also known as sFlt-1), an anti-angiogenic protein, whose increased expression has been associated with, and indeed may be diagnostic for, PE [35-37]. The syncytial knots in PE/FGR also show a decrease in the anti-apoptotic BCL-2 protein and an increase in cell death, as measured by TUNEL staining [38]. The increase in cell death has been attributed to caspase-independent apoptosis [39], increased levels of endoplasmic reticulum stress [40], as well as excessive autophagy [41,42]. The increase in syncytial knots and cell death leads to discontinuity of the syncytium, resulting in leakage of fetal proteins into maternal circulation [43]. It also likely leads to increased thickness (~two-fold) of the vasculosyncytial membrane, the exchange interface of chorionic villi, thus negatively affecting fetal growth [44]. Finally, at least in early-onset PE, this increase in syncytial knots and apoptosis is also associated with an increase in STB turnover/shedding, with over two-fold increase in circulating STB-derived microparticles, compared to gestational age-matched control cases [45].

At the molecular level, these alterations in STB morphology and function have been attributed to alterations in the GCM1-SYNCYTIN pathway, regulating syncytialization and STB organization. Specifically, both GCM1 and SYNCYTIN-1 proteins have been found to be reduced in PE placentae [46,47], with SYNCYTIN-1 also being mis-localized to the apical, instead of the basal, membrane in STB [47]. Hypoxia has been proposed as the potential mechanism for GCM1 downregulation, acting through suppression of the PI3K-AKT signaling pathway to activation of GSK3β, with subsequent phosphorylation of GCM1, targeting it for ubiquitination and degradation [48]. A reduction in GCM1 leads to a decrease in SYNCYTIN expression, resulting, in turn, in reduced fusion and an altered syncytium.

Other functions that have been attributed to SYNCYTIN include both promotion of cell proliferation [49] and inhibition of apoptosis [39]. However, while the latter is consistent with the STB phenotype noted in PE, PE-associated alterations in villous CTB proliferation have been somewhat controversial. Intuitively, it is easy to assume that accelerated maturation must be accompanied by a loss in CTB proliferation. However, results of studies evaluating CTB proliferation in PE/FGR range from no differences in CTB proliferation [50,51], region-specific alterations in CTB proliferation [52], to an increase in CTB proliferation attributed to a damaged

syncytium in need of repair and regeneration [53,54]. Given the heterogeneity of PE and FGR, as recently elaborated on by genome-wide expression profiling of placental tissues associated with these complications [20-23], it is possible that distinct subclasses of these diseases have different alterations at the cellular level, including differences in CTB proliferation, depending on the underlying pathophysiology.

Abnormalities of extravillous trophoblast in PE/FGR

Extravillous trophoblast (EVT) differentiate from CTB precursor cells within anchoring chorionic villi at the basal plate. This process occurs within trophoblast cell columns, where CTB gradually lose expression of EGFR and gain HLAG expression [6]. This process occurs early in gestation, in a relatively hypoxic microenvironment [55] and, in fact, is regulated by the hypoxia-inducible complex (HIF), at least in its initial transition from CTB to EVT [6]. HIF, in turn, induces expression of transcription factors ASCL2 and TEAD2, which are specifically expressed in EVT in the human placenta [6]. Hypoxia also induces NOTCH1, which inhibits expression of transcription factors involved in CTB self-renewal, including TEAD4 and TP63, represses syncytialization, and promotes proliferation and survival of EVT precursor cells [56]. GCM1 is another transcription factor induced during EVT differentiation, although it does not appear to be regulated by HIF in this context [6]. Because GCM1 is also involved in STB differentiation of CTB progenitor cells [24-26], cellular context/microenvironment likely plays a key role in distinguishing which lineage is induced downstream of GCM1 expression.

Aside from hypoxia/HIF, two other pathways known to be involved in EVT differentiation include epithelial-mesenchymal transition (EMT) and WNT signaling. As the cells move distally within the column, they lose their proliferative potential and undergo what has been described as a "partial EMT," losing some (but not all) of their epithelial markers, and gaining some mesenchymal markers [57,58]. This phenotype is accompanied by an induction of ZEB2 [57] and TEAD2 [6], as mentioned above, both of which are transcription factors known to be involved in EMT [59,60]. This process also involves a switch in cell surface integrins, from ITGA6 in CTB, to ITGA5 in cell column/immature EVT, and ITGA1 in mature EVT [61], acquiring a vascular phenotype, including induction of VE-Cadherin, VCAM-1, and PECAM [62], as well as cytoskeletal remodeling [6,57]. WNT signaling is also important for EVT differentiation, requiring to be turned off during the initial transition from CTB to EVT, but subsequently needed to be reactivated, acting through the transcription factor TCF4 to promote EVT maturation [63,64].

EVTs are more difficult to evaluate, as they are left behind, for the most part, following delivery of the placenta (those in the decidua basalis). Nevertheless, evaluation of decidual vessels present within the fetal membranes (those in the decidua parietalis) are thought to reflect the general state of maternal uterine vessels, and by inference, the functional status of endovascular EVT, for that particular pregnancy [1,2]. At the same time, evaluation of the basal plate EVT (those in the decidua basalis--at the maternal surface of the placental disc) provides at least some insight into the differentiation and function of interstitial EVT [1,2]. Combined, these evaluations have identified lesions which suggest that both PE and FGR, particularly in pre-term and severe cases, are associated with abnormalities of EVT differentiation, vascular remodeling, and invasion [1,2]. These lesions include decidual vasculopathy, characterized by hypertrophy/hyperplasia of the muscular layer of the decidual arterioles, perivascular chronic inflammation, fibrinoid necrosis, and/or infiltration by foamy macrophages [2]. The presence of decidual vasculopathy, alongside multifocal infarction, within PE/FGR placentae is highly

suggestive of a defect in endovascular EVT function, which leads to lack of proper maternal blood flow to the placenta [2]. In addition, at the basal plate, an increase in immature interstitial EVT has been confirmed by increased markers of proliferation and reduced immunostaining for human placental lactogen (HPL) [65].

Additional insight into EVT differentiation and function has come from placental bed biopsies, performed post-delivery for research purposes. These studies have pointed to limited vascular remodeling in both PE and FGR placentae, with the resulting physiologic conversion of the arteries being confined to the decidual portions of these vessels, and not extending into the myometrium [66-68]. The absence of remodeling in these biopsies has also been associated with a decrease in number of mature, HPL^+ interstitial EVT [69], but with an increase in placental bed giant cells, though a specific origin for these cells has yet to be confirmed [70]. Evaluation of these biopsies by immunohistochemistry has also revealed a lack of integrin switching, as well as failure to acquire a vascular adhesion phenotype, with significantly reduced expression of VE-Cadherin and VCAM-1 in endovascular and interstitial EVT, respectively [71].

More recently, more specific markers for differentiated EVT subtypes have been identified and characterized in context of PE/FGR. One example is PLAC8, which has been identified as specific to interstitial EVT [72]. PLAC8 is an actin-associated protein which promotes migration and invasion of these cells, through activation of RAC1 and CDC42 [72]. However, its expression was found to be elevated in interstitial EVT of PE placentae, speculated to be a compensatory response to the shallow invasion of these cells in PE [72]. A second example is LAIR2 (Leukocyte-Associated Immunogloubin-like Receptor-2), which appears to be specific to the endovascular and vascular plug EVT [73]. LAIR2 has been noted to be decreased in chorionic villus samples of placentae associated with PE development later in pregnancy [74]; however, it is not clear whether this finding is significant, particularly if this protein is in fact specifically expressed in endovascular EVT, as CVS would be unlikely to capture this cell population. Nevertheless, a decrease in LAIR2 in the placental bed of PE patients, if measured, may be explained by an increase in apoptosis in endovascular EVT, which has reported in context of early-onset PE accompanied by FGR [75]. Unfortunately, similar to the LAIR2 study above, many studies have used villous tissue from PE/FGR placentae to evaluate alterations in pathways involved in EVT differentiation, including EMT [76,77] and WNT signaling [78,79]. While this is understandable, given the paucity of material, particularly of placental bed biopsies, ideal for these studies, these results preclude definitive conclusions on the role of alterations of these pathways in the pathogenesis of PE/FGR.

Conclusions and Gaps in Knowledge

While much has been learned about pathways regulating human trophoblast differentiation over the past ~30 years, many questions remain. In particular, in comparison to mouse, where studies have led to the identification of numerous trophoblast subtypes in both the labyrinth and junctional zones (equivalent to villous and extravillous compartments in the human placenta) [80,81], we know very little about the heterogeneity of both CTB and EVT subtypes. Recent studies using single cell RNAseq are beginning to shed light in this area [9-11]; however, significant work remains, on identification and localization of different cell types, both within normal development of the placenta and in the setting of diseases such as PE and FGR. Perhaps the most important point for future studies would be to first assess the expression pattern of an individual gene product within the placenta across gestation, prior to evaluation of its expression (by qPCR, western blot, or other means) using banked tissues from diseased placentae, most of

which consist of only villous portions of this organ. Alternatively, immunohistochemistry/in-situ hybridization can provide a means to evaluate cell- or region-specific expression, albeit in a less quantitative manner. In addition, detailed molecular studies of pathways involved in EVT differentiation, specifically evaluating both interstitial and endovascular EVT in the setting of PE/FGR, are currently scant and require widely available perinatal tissue banks which include placental bed biopsies. Finally, functional analysis of these pathways requires improved *in vitro* systems for studying human trophoblast differentiation, including recently-established trophoblast stem cells and organoids, as well as iPSC-based models of human placental disease [3,82,83]. With the availability of the above resources, this field is poised to significantly advance over the next few decades, accompanying development of useful biomarkers and therapies for diagnosis and treatment of placenta-based disorders of pregnancy.

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Author contributions

OF and CT did the preliminary literature search. CN performed the immunohistochemistry which contributed to the generation of figures. OF and MMP wrote the manuscript text.

References

1) K. Benirschke, G.J. Burton, R.N. Baergen. Pathology of the Human Placenta. Springer-Verlag Berlin Heidelberg, Berlin, Heidelberg, 2012.

2) R.W. Redline, T.K. Boyd, D.J. Roberts. Placental and Gestational Pathology. Cambridge University Press. Cambridge, UK. 2018.

3) M. Knöfler, S. Haider, L. Saleh, J. Pollheimer, T.K.J.B. Gamage, J. James. Human placenta and trophoblast development: key molecular mechanisms and model systems. Cell Mol Life Sci 76 (18) (2019) 3479-3496.

4) S.J. Fisher. Why is placentation abnormal in preeclampsia? Am J Obstet Gynecol 213 (4 Suppl) (2015) S115-22.

5) G.J. Burton, E. Jauniaux. Pathophysiology of placental-derived fetal growth restriction. Am J Obstet Gynecol 218 (2S) (2018) S745-S761.

6) A.K. Wakeland, F. Soncin, M. Moretto-Zita, C.W. Chang, M. Horii, D. Pizzo, K.K. Nelson, L.C. Laurent, M.M. Parast. Hypoxia Directs Human Extravillous Trophoblast Differentiation in a Hypoxia-Inducible Factor-Dependent Manner. Am J Pathol 187 (4) (2017) 767-780.

7) R. Pijnenborg, J.M. Bland, W.B. Robertson, G. Dixon, I. Brosens. The pattern of interstitial trophoblastic invasion of the myometrium in early human pregnancy. Placenta 2 (1981) 303-316.

8) Y. Lee, K.R. Kim, F. McKeon, A. Yang, T.K. Boyd, C.P. Crum, M.M. Parast. A unifying concept of trophoblastic differentiation and malignancy defined by biomarker expression. Hum Pathol 38 (7) (2007) 1003-13.

9) H. Suryawanshi, P. Morozov, A. Straus, N. Sahasrabudhe, K.E.A. Max, A. Garzia, M. Kustagi, T. Tuschl, Z. Williams. A single-cell survey of the human first-trimester placenta and decidua. Sci Adv 4 (10) (2018) eaau4788.

10) Y. Liu, X. Fan, R. Wang, X. Lu, Y.L. Dang, H. Wang, H.Y. Lin, C. Zhu, H. Ge, J.C. Cross, H. Wang. Single-cell RNA-seq reveals the diversity of trophoblast subtypes and patterns of differentiation in the human placenta. Cell Res 28 (8) (2018) 819-832.

11) R. Vento-Tormo, M. Efremova, R.A. Botting, M.Y. Turco, M. Vento-Tormo, K.B. Meyer, J.E. Park, E. Stephenson, K. Polański, A. Goncalves, L. Gardner, S. Holmqvist, J. Henriksson, A. Zou, A.M. Sharkey, B. Millar, B. Innes, L. Wood, A. Wilbrey-Clark, R.P. Payne, M.A. Ivarsson, S. Lisgo, A. Filby, D.H. Rowitch, J.N. Bulmer, G.J. Wright, M.J.T. Stubbington, M. Haniffa, A. Moffett, S.A. Teichmann. Single-cell reconstruction of the early maternal-fetal interface in humans. Nature 563 (7731) (2018) 347-353.

12) F. Soncin, M. Khater, C. To, D. Pizzo, O. Farah, A. Wakeland, K. Arul Nambi Rajan, K.K. Nelson, C.W. Chang, M. Moretto-Zita, D.R. Natale, L.C. Laurent, M.M. Parast. Comparative

345 analysis of mouse and human placentae across gestation reveals species-specific regulators of
346 placental development. Development 145 (2) (2018) pii: dev156273.
347
348 13) N. Ishihara, H. Matsuo, H. Murakoshi, J. Laoag-Fernandez, T. Samoto, T. Maruo. Changes
349 in proliferative potential, apoptosis and Bcl-2 protein expression in cytotrophoblasts and
350 syncytiotrophoblast in human placenta over the course of pregnancy. Endocrine J 47 (3) (2000)
351 317-327.
352
353 14) E. Eiland, C. Nzerue, M. Faulkner. Preeclampsia 2012. J Pregnancy 2012 (2012) 586578.
354
355 15) American College of Obstetricians and Gynecologists; Task Force on Hypertension in
356 Pregnancy. Hypertension in Pregnancy: Report of the American College of Obstetricians and
357 Gynecologists' Task Force on Hypertension in Pregnancy. Obstet Gynecol 122 (5) (2013) 1122-
358 31.
359
360 16) J.T. Henderson, J.H. Thompson, B.U. Burda, A. Cantor. Preeclampsia Screening: Evidence
361 Report and Systematic Review for the US Preventive Services Task Force. JAMA 317 (16)
362 (2017) 1668-1683.
363
364 17) C.W. Redman, A.C. Staff. Preeclampsia, biomarkers, syncytiotrophoblast stress, and
365 placental capacity. Am J Obstet Gynecol 213 (4 Suppl) (2015) S9-11.
366
367 18) M.C. Audette, J.C. Kingdom. Screening for fetal growth restriction and placental
368 insufficiency. Semin Fetal Neonatal Med 23 (2) (2018) 119-125.
369
370 19) L. Kroener, E.T. Wang, M.D. Pisarska. Predisposing Factors to Abnormal First Trimester
371 Placentation and the Impact on Fetal Outcomes. Semin Reprod Med 34(1) (2016) 27-35.
372
373 20) K. Leavey, S.A. Bainbridge, B.J. Cox. Large scale aggregate microarray analysis reveals
374 three distinct molecular subclasses of human preeclampsia. PLoS One 10 (2) (2015) e0116508.
375
376 21) K. Leavey, S.J. Benton, D. Grynspan, J.C. Kingdom, S.A. Bainbridge, B.J. Cox.
377 Unsupervised Placental Gene Expression Profiling Identifies Clinically Relevant Subclasses of
378 Human Preeclampsia. Hypertension 68 (1) (2016) 137-47.
379
380 22) S.J. Benton, K. Leavey, D. Grynspan, B.J. Cox, S.A. Bainbridge. The clinical heterogeneity
381 of preeclampsia is related to both placental gene expression and placental histopathology. Am J
382 Obstet Gynecol 219 (6) (2018) 604.e1-604.e25.
383
384 23) I. Gibbs, K. Leavey, S.J. Benton, D. Grynspan, S.A. Bainbridge, B.J. Cox. Placental
385 transcriptional and histologic subtypes of normotensive fetal growth restriction are comparable
386 to preeclampsia. Am J Obstet Gynecol 220 (1) (2019) 110.e1-110.e21.
387
388 24) D. Baczyk, A. Satkunaratnam, B. Nait-Oumesmar, B. Huppertz, J.C. Cross, J.C. Kingdom.
389 Complex patterns of GCM1 mRNA and protein in villous and extravillous trophoblast cells of
390 the human placenta. Placenta 25 (6) (2004) 553-9.

25) C. Lin, M. Lin, H. Chen. Biochemical characterization of the human placental transcription factor GCMa/1. Biochem Cell Biol 83 (2) (2005) 188-95.

26) D. Baczyk, S. Drewlo, L. Proctor, C. Dunk, S. Lye, J. Kingdom. Glial cell missing-1 transcription factor is required for the differentiation of the human trophoblast. Cell Death Differ 16 (5) (2009) 719-27.

27) S. Mi, X. Lee, X. Li, G.M. Veldman, H. Finnerty, L. Racie, E. LaVallie, X.Y. Tang, P. Edouard, S. Howes, J.C. Keith Jr, J.M. McCoy. Syncytin is a captive retroviral envelope protein involved in human placental morphogenesis. Nature 403 (6771) (2000) 785-9.

28) X. Lu, R. Wang, C. Zhu, H. Wang, H.Y. Lin, Y. Gu, J.C. Cross, H. Wang. Fine-Tuned and Cell-Cycle-Restricted Expression of Fusogenic Protein Syncytin-2 Maintains Functional Placental Syncytia. Cell Rep 21 (5) (2017) 1150-1159.

29) K. Asklund, L. Chamley. Trophoblast deportation part I: review of the evidence demonstrating trophoblast shedding and deportation during human pregnancy. Placenta 32 (10) (2011) 716–723.

30) K. Loukeris, R. Sela, R.N. Baergen. Syncytial knots as a reflection of placental maturity: reference values for 20 to 40 weeks' gestational age. Pediatr Dev Pathol 13 (4) (2010) 305-309.

31) N.M. Fogarty, A.C. Ferguson-Smith, G.J. Burton. Syncytial knots (Tenney-Parker changes) in the human placenta: evidence of loss of transcriptional activity and oxidative damage. Am J Pathol 183 (1) (2013) 144-152.

32) T.Y. Khong, E.E. Mooney, I. Ariel, N.C. Balmus, T.K. Boyd, M.A. Brundler, H. Derricott, M.J. Evans, O.M. Faye-Petersen, J.E. Gillan, A.E. Heazell, D.S. Heller, S.M. Jacques, S. Keating, P. Kelehan, A. Maes, E.M. McKay, T.K. Morgan, P.G. Nikkels, W.T. Parks, R.W. Redline, I. Scheimberg, M.H. Schoots, N.J. Sebire, A. Timmer, G. Turowski, J.P. van der Voorn, I. van Lijnschoten, S.J. Gordijn. Sampling and Definitions of Placental Lesions: Amsterdam Placental Workshop Group Consensus Statement. Arch Pathol Lab Med 140 (7) (2016) 698-713.

33) B. Tenney, F. Parker. The placenta in toxemia of pregnancy. Am J Obstet Gynecol 39 (6) (1940) 1000–1005.

34) G.J. Burton, A.W. Woods, E. Jauniaux, J.C. Kingdom. Rheological and physiological consequences of conversion of the maternal spiral arteries for uteroplacental blood flow during human pregnancy. Placenta 30 (6) (2009) 473-82.

35) V. Taché, D.Y. LaCoursiere, A. Saleemuddin, M.M. Parast. Placental expression of vascular endothelial growth factor receptor-1/soluble vascular endothelial growth factor receptor-1 correlates with severity of clinical preeclampsia and villous hypermaturity. Hum Pathol 42 (9) (2011) 1283-8.

36) S.E. Maynard, J.Y. Min, J. Merchan, K.H. Lim, J. Li, S. Mondal, T.A. Libermann, J.P. Morgan, F.W. Sellke, I.E. Stillman, F.H. Epstein, V.P. Sukhatme, S.A. Karumanchi. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. J Clin Invest 111 (5) (2003) 649-58.

37) R.J. Levine, S.E. Maynard, C. Qian, K.H. Lim, L.J. England, K.F. Yu, E.F. Schisterman, R. Thadhani, B.P. Sachs, F.H. Epstein, B.M. Sibai, V.P. Sukhatme, S.A. Karumanchi. Circulating angiogenic factors and the risk of preeclampsia. N Engl J Med 350 (7) (2004) 672-83.

38) N. Ishihara, H. Matsuo, H. Murakoshi, J.B. Laoag-Fernandez, T. Samoto, T. Maruo. Increased apoptosis in the syncytiotrophoblast in human term placentas complicated by either preeclampsia or intrauterine growth retardation. Am J Obstet Gynecol 186 (1) (2002) 158-66.

39) Q. Huang, H. Chen, F. Wang, B.C. Brost, J. Li, Y. Gao, Z. Li, Y. Gao, S.W. Jiang. Reduced syncytin-1 expression in choriocarcinoma BeWo cells activates the calpain1-AIF-mediated apoptosis, implication for preeclampsia. Cell Mol Life Sci 71 (16) (2014) 3151–3165.

40) H.W. Yung, S. Calabrese, D. Hynx, B.A. Hemmings, I. Cetin, D.S. Charnock-Jones, G.J. Burton. Evidence of placental translation inhibition and endoplasmic reticulum stress in the etiology of human intrauterine growth restriction. Am J Pathol 173 (2) (2008) 451-62.

41) S.Y. Oh, S.J. Choi, K.H. Kim, E.Y. Cho, J.H. Kim, C.R. Roh. Autophagy-related proteins, LC3 and Beclin-1, in placentas from pregnancies complicated by preeclampsia. Reprod Sci 15 (9) (2008) 912–920.

42) T.H. Hung, S.F. Chen, L.M. Lo, M.J. Li, Y.L. Yeh, T.T. Hsieh. Increased autophagy in placentas of intrauterine growth-restricted pregnancies. PLoS One 7 (2012) e40957.

43) C.S. Roland, J. Hu, C.E. Ren, H. Chen, J. Li, M.S. Varvoutis, L.W. Leaphart, D.B. Byck, X. Zhu, S.W. Jiang. Morphological changes of placental syncytium and their implications for the pathogenesis of preeclampsia. Cell Mol Life Sci 73 (2) (2016) 365-76.

44) K.D. Sankar, P.S. Bhanu, S. Kiran, B.A. Ramakrishna, V. Shanthi. Vasculosyncytial membrane in relation to syncytial knots complicates the placenta in preeclampsia: a histomorphometrical study. Anat Cell Biol 45 (2) (2012) 86–91.

45) D. Goswami, D.S. Tannetta, L.A. Magee, A. Fuchisawa, C.W. Redman, I.L. Sargent, P. von Dadelszen. Excess syncytiotrophoblast microparticle shedding is a feature of early-onset pre-eclampsia, but not normotensive intrauterine growth restriction. Placenta 27 (1) (2006) 56-61.

46) C.P. Chen, C.Y. Chen, Y.C. Yang, T.H. Su, H. Chen. Decreased placental GCM1 (Glial Cells Missing) gene expression in preeclampsia. Placenta 25 (5) (2004) 413-421

47) X. Lee, J.C. Keith Jr, N. Stumm, I. Moutsatsos, J.M. McCoy, C.P. Crum, D. Genest, D. Chin, C. Ehrenfels, R. Pijnenborg, F.A. van Assche, S. Mi. Downregulation of placental syncytin expression and abnormal protein localization in pre-eclampsia. Placenta 22 (10) (2001) 808-12.

48) M.H. Chiang, F.Y. Liang, C.P. Chen, C.W. Chang, M.L. Cheong, L.J. Wang, C.Y. Liang, F.Y. Lin, C.C. Chou, H. Chen. Mechanism of hypoxia-induced GCM1 degradation: implications for the pathogenesis of preeclampsia. J Biol Chem 284 (26) (2009) 17411-9.

49) Q. Huang, J. Li, F. Wang, M.T. Oliver, T. Tipton, Y. Gao, S.W. Jiang. Syncytin-1 modulates placental trophoblast cell proliferation by promoting G1/S transition. Cell Signal 25 (4) (2013) 1027–1035.

50) U. Jeschke, B. Schiessl, I. Mylonas, S. Kunze, C. Kuhn, S. Schulze, K. Friese, D. Mayr. Expression of the proliferation marker Ki-67 and of p53 tumor protein in trophoblastic tissue of preeclamptic, HELLP, and intrauterine growth-restricted pregnancies. Int J Gynecol Pathol 25 (4) (2006) 354-60.

51) N. Vedmedovska, D. Rezeberga, U. Teibe, I. Melderis, G.G. Donders. Placental pathology in fetal growth restriction. Eur J Obstet Gynecol Reprod Biol 155 (2011) 36-40.

52) G. Unek, A. Ozmen, I. Mendilcioglu, M. Simsek, E.T. Korgun. The expression of cell cycle related proteins PCNA, Ki67, p27 and p57 in normal and preeclamptic human placentas. Tissue Cell 46 (3) (2014) 198-205.

53) H. Arnholdt, F. Meisel, K. Fandrey, U. Löhrs. Proliferation of villous trophoblast of the human placenta in normal and abnormal pregnancies. Virchows Arch B Cell Pathol Incl Mol Pathol 60 (6) (1991) 365-72.

54) R. Madazli, A. Somunkiran, Z. Calay, S. Ilvan, M.F. Aksu. Histomorphology of the placenta and the placental bed of growth restricted fetuses and correlation with the Doppler velocimetries of the uterine and umbilical arteries. Placenta 24 (2003) 510-6.

55) E. Jauniaux, A.L. Watson, J. Hempstock, Y.P. Bao, J.N. Skepper, G.J. Burton. Onset of maternal arterial blood flow and placental oxidative stress. A possible factor in human early pregnancy failure. Am J Pathol 157 (6) (2000) 2111-22.

56) S. Haider, G. Meinhardt, L. Saleh, C. Fiala, J. Pollheimer, M. Knöfler. Notch1 controls development of the extravillous trophoblast lineage in the human placenta. Proc Natl Acad Sci U S A 113 (48) (2016) E7710-E7719.

57) S. DaSilva-Arnold, J.L. James, A. Al-Khan, S. Zamudio, N.P. Illsley. Differentiation of first trimester cytotrophoblast to extravillous trophoblast involves an epithelial-mesenchymal transition. Placenta 36 (12) (2015) 1412-1418.

58) J.E. Davies, J. Pollheimer, H.E. Yong, M.I. Kokkinos, B. Kalionis, M. Knöfler, P. Murthi. Epithelial-mesenchymal transition during extra- villous trophoblast differentiation. Cell Adh Migr 12 (2016) 1e12.

59) C. Vandewalle, J. Comijn, B. De Craene, P. Vermassen, E. Bruyneel, H. Andersen, E. Tulchinsky, F. Van Roy, G. Berx. SIP1/ZEB2 induces EMT by repressing genes of different epithelial cell-cell junctions, Nucleic Acids Res 33 (20) (2005) 6566-6578.

60) H. Zhang, C.Y. Liu, Z.Y. Zha, B. Zhao, J. Yao, S. Zhao, Y. Xiong, Q.Y. Lei, K.L. Guan. TEAD transcription factors mediate the function of TAZ in cell growth and epithelial-mesenchymal transition. J Biol Chem 284 (2009) 13355e13362.

61) C.H. Damsky, C. Librach, K.H. Lim, M.L. Fitzgerald, M.T. McMaster, M. Janatpour, Y. Zhou, S.K. Logan, S.J. Fisher. Integrin switching regulates normal trophoblast invasion. Development 120 (12) (1994) 3657-66.

62) Y. Zhou, S.J. Fisher, M. Janatpour, O. Genbacev, E. Dejana, M. Wheelock, C.H. Damsky. Human cytotrophoblasts adopt a vascular phenotype as they differentiate. A strategy for successful endovascular invasion? J Clin Invest 99 (9) (1997) 2139-51.

63) S. Haider, G. Meinhardt, L. Saleh, V. Kunihs, M. Gamperl, U. Kaindl, A. Ellinger, T.R. Burkard, C. Fiala, J. Pollheimer, S. Mendjan, P.A. Latos, M. Knöfler. Self-Renewing Trophoblast Organoids Recapitulate the Developmental Program of the Early Human Placenta. Stem Cell Reports 11 (2) (2018) 537-551.

64) G. Meinhardt, S. Haider, P. Haslinger, K. Proestling, C. Fiala, J. Pollheimer, M. Knöfler. Wnt-dependent T-cell factor-4 controls human extravillous trophoblast motility. Endocrinology 155 (5) (2014) 1908-20.

65) R.W. Redline, P. Patterson. Preeclampsia is associated with an excess of proliferative immature intermediate trophoblast. Hum Pathol 26 (6) (1995) 594-600.

66) I.A. Brosens, W.B. Robertson, H.G. Dixon. The role of the spiral arteries in the pathogenesis of preeclampsia. Obstet Gynecol Annu 1 (1972) 177-91.

67) I. Brosens, H.G. Dixon, W.B. Robertson. Fetal growth retardation and the arteries of the placental bed. Br J Obstet Gynaecol 84 (9) (1977) 656-63.

68) T.Y. Khong, F. De Wolf, W.B. Robertson, I. Brosens. Inadequate maternal vascular response to placentation in pregnancies complicated by pre-eclampsia and by small-for-gestational age infants. Br J Obstet Gynaecol 93 (10) (1986) 1049–1059

69) S. Gosseye, F. van der Veen. HPL-positive infiltrating trophoblastic cells in normal and abnormal pregnancy. Eur J Obstet Gynecol Reprod Biol 44 (2) (1992) 85-90.

70) G. Gerretsen, H.J. Huisjes, M.J. Hardonk, J.D. Elema. Trophoblast alterations in the placental bed in relation to physiological changes in spiral arteries. Br J Obstet Gynaecol 90 (1) (1983) 34-9.

71) Y. Zhou, C.H. Damsky, S.J. Fisher. Preeclampsia is associated with failure of human cytotrophoblasts to mimic a vascular adhesion phenotype. One cause of defective endovascular invasion in this syndrome? J Clin Invest 99 (9) (1997) 2152-2164.

72) W.L. Chang, Y.W. Liu, Y.L. Dang, X.X. Jiang, H. Xu, X. Huang, Y.L. Wang, H. Wang, C. Zhu, L.Q. Xue, H.Y. Lin, W. Meng, H. Wang. PLAC8, a new marker for human interstitial extravillous trophoblast cells, promotes their invasion and migration. Development 145 (2) (2018) pii: dev148932.

73) S.A. Founds, B. Fallert-Junecko, T.A. Reinhart, W.T. Parks. LAIR2-expressing extravillous trophoblasts associate with maternal spiral arterioles undergoing physiologic conversion. Placenta 34 (2013) 248-255.

74) S.A. Founds, Y.P. Conley, J.F. Lyons-Weiler, A. Jeyabalan, W.A. Hogge, K.P. Conrad. Altered global gene expression in first trimester placentas of women destined to develop preeclampsia. Placenta 30 (1) (2009) 15e24.

75) M. Kadyrov, J.C. Kingdom, B. Huppertz. Divergent trophoblast invasion and apoptosis in placental bed spiral arteries from pregnancies complicated by maternal anemia and early-onset preeclampsia/intrauterine growth restriction. Am J Obstet Gynecol 194 (2) (2006) 557-63.

76) K. Blechschmidt, I. Mylonas, D. Mayr, B. Schiessl, S. Schulze, K.F. Becker, U. Jeschke. Expression of E-cadherin and its repressor snail in placental tissue of normal, preeclamptic and HELLP pregnancies. Virchows Arch 450 (2) (2007) 195-202.

77) L. Fedorova, C. Gatto-Weis, S. Smaili, N. Khurshid, J.I. Shapiro, D. Malhotra, T. Horrigan. Down-regulation of the transcription factor snail in the placentas of patients with preeclampsia and in a rat model of preeclampsia. Reprod Biol Endocrinol 10 (2012) 15.

78) X. Wang, Z. Zhang, X. Zeng, J. Wang, L. Zhang, W. Song, Y. Shi. Wnt/β-catenin signaling pathway in severe preeclampsia. J Mol Histol 49 (3) (2018) 317-327.

79) L. Huang, H. Ying, Z. Chen, Y.L. Zhu, Y. Gu, L. Hu, D. Chen, N. Zhong. Down-regulation of DKK1 and Wnt1/β-catenin pathway by increased homeobox B7 resulted in cell differentiation suppression of intrauterine fetal growth retardation in human placenta. Placenta 80 (2019) 27-35.

80) D.G. Simmons, D.R.C. Natale, V. Begay, M. Hughes, A. Leutz, J.C. Cross. Early patterning of the chorion leads to the trilaminar trophoblast cell structure in the placental labyrinth. Development 135 (2008) 2083-2091.

81) D.G. Simmons, A.L. Fortier, J.C. Cross. Diverse subtypes and developmental origins of trophoblast giant cells in the mouse placenta. Dev Biol 304 (2007) 567-578.

82) H. Okae, H. Toh, T. Sato, H. Hiura, S. Takahashi, K. Shirane, Y. Kabayama, M. Suyama, H. Sasaki, T. Arima. Derivation of Human Trophoblast Stem Cells. Cell Stem Cell 22 (1) (2018) 50-63.e6.

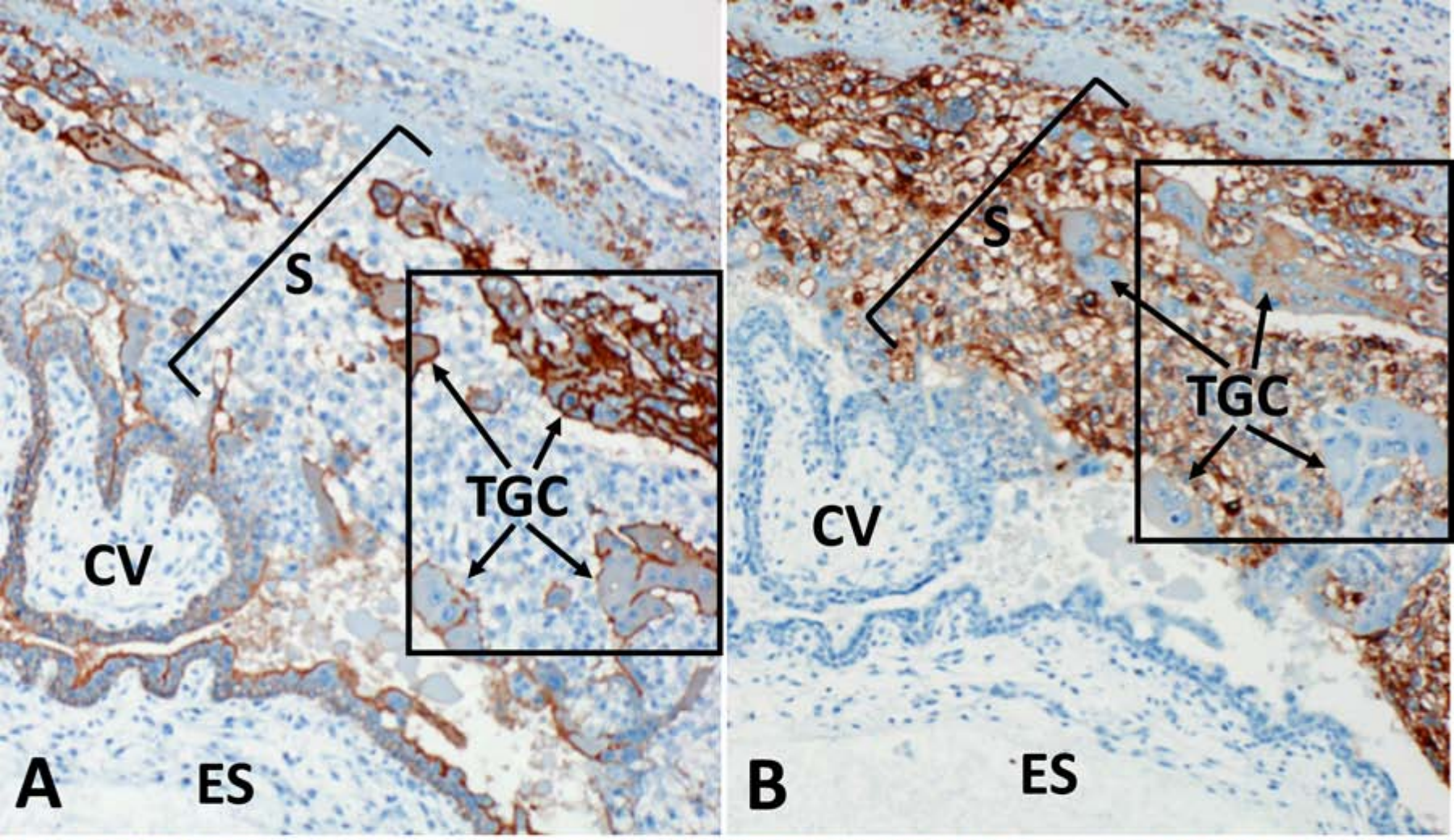
83) M.A. Sheridan, Y. Yang, A. Jain, A.S. Lyons, P. Yang, S.R. Brahmasani, A. Dai, Y. Tian, M.R. Ellersieck, G. Tuteja, D.J. Schust, L.C. Schulz, T. Ezashi, R.M. Roberts. Early onset preeclampsia in a model for human placental trophoblast. Proc Natl Acad Sci U S A 116 (10) (2019) 4336-4345.

Figure Legends

Figure 1. Sequential sections of an archived day-16 human embryo stained with antibodies to EGFR (**A, C**) or HLAG (**B, D**). Formalin-fixed paraffin embedded (FFPE) tissue was sectioned and stained with a rabbit monoclonal antibody against EGFR (clone 5B7, Ventana/Roche) or a mouse monoclonal antibody against HLAG (4H84 clone, Abcam), using a Ventana Discovery Ultra automated immunostainer with standard antigen retrieval and reagents as per the manufacturer's protocol. Note that EGFR highlights both villous trophoblast surrounding the embryonic sac (ES) and primary chorionic villi (CV), as well as trophoblast giant cells (TGC) embedded within the trophoblastic shell (S), while HLAG predominantly highlights the mononuclear trophoblast within the trophoblastic shell. Immunostaining is shown in brown with blue (hematoxylin) counterstain. C and D represent magnification of the trophoblastic shell area with TGC, bracketed in a rectangular area in A and B, respectively. Magnification of 40x (for A and B) and 80x (for C and D).

Figure 2. Human placenta at term double-stained with antibodies to EGFR (green) and HLAG (red), counterstained with DAPI (blue). FFPE sections from 12 different term placentas were de-paraffinized and rehydrated, and antigen retrieval was performed using heat and antigen retrieval buffer (Dako) for 20 minutes. Tissues were incubated with the same primary antibodies as used for immunohistochemistry shown in Figure 1, then visualized by Alexa 488- or Alexa 594-conjugated secondary antibodies (Invitrogen) and counter-stained with the nuclear stain, DAPI. **A)** Chorionic plate (near the fetal surface), showing a layer of $EGFR^+$ cytotrophoblast (CTB) immediately below the amniotic mesenchyme (AM), with $HLAG^+$ cells emanating from the CTB layer, protruding into the intervillous space (IVS). **B)** Basal plate (near the maternal surface), showing $EGFR^+$ syncytiotrophoblast covering chorionic villi (CV, top right), below which are multiple layers of mature, $HLAG^+$ cells. **C)** An intraplacental trophoblast island within the placental disc, where a chorionic villus remnant (CV) gets surrounded by perivillous fibrin resulting in differentiation of CTB into $HLAG^+$ EVT. **D)** An EGFR/HLAG double-positive multinucleated cell (arrow) at the basal plate, surrounded by $HLAG^+$ EVT and unstained decidual cells. Magnification of 80x for A-D.

Figure 3. Cytotrophoblast heterogeneity in early gestation (6-week gestational age) human placenta. FFPE sections from 5 different placentas were stained, either with antibodies against TP63 (mouse monoclonal antibody specific to p40, BC28 clone, Biocare Medical), EGFR (same antibody as in Figure 1), and CD31 (rabbit monoclonal, ab76533, Abcam) (shown in A) , or against CDX2 (rabbit monoclonal, ab76541, Abcam) (shown in B), using a Ventana Discovery Ultra automated immunostainer with standard antigen retrieval and reagents as per the manufacturer's protocol. **A)** Section showing triple staining for TP63 (brown), EGFR (magenta), and CD31 (yellow-green), counter-stained with hematoxylin (blue). Note that TP63 is uniformly expressed in the CTB layer (trophoblast layer adjacent to the villous stroma/VS). **B)** Section showing staining for CDX2 (brown), counter-stained with hematoxylin (blue). Note stretches of CTB that lack CDX2 staining (yellow arrowheads). Magnification of 80x for A-B.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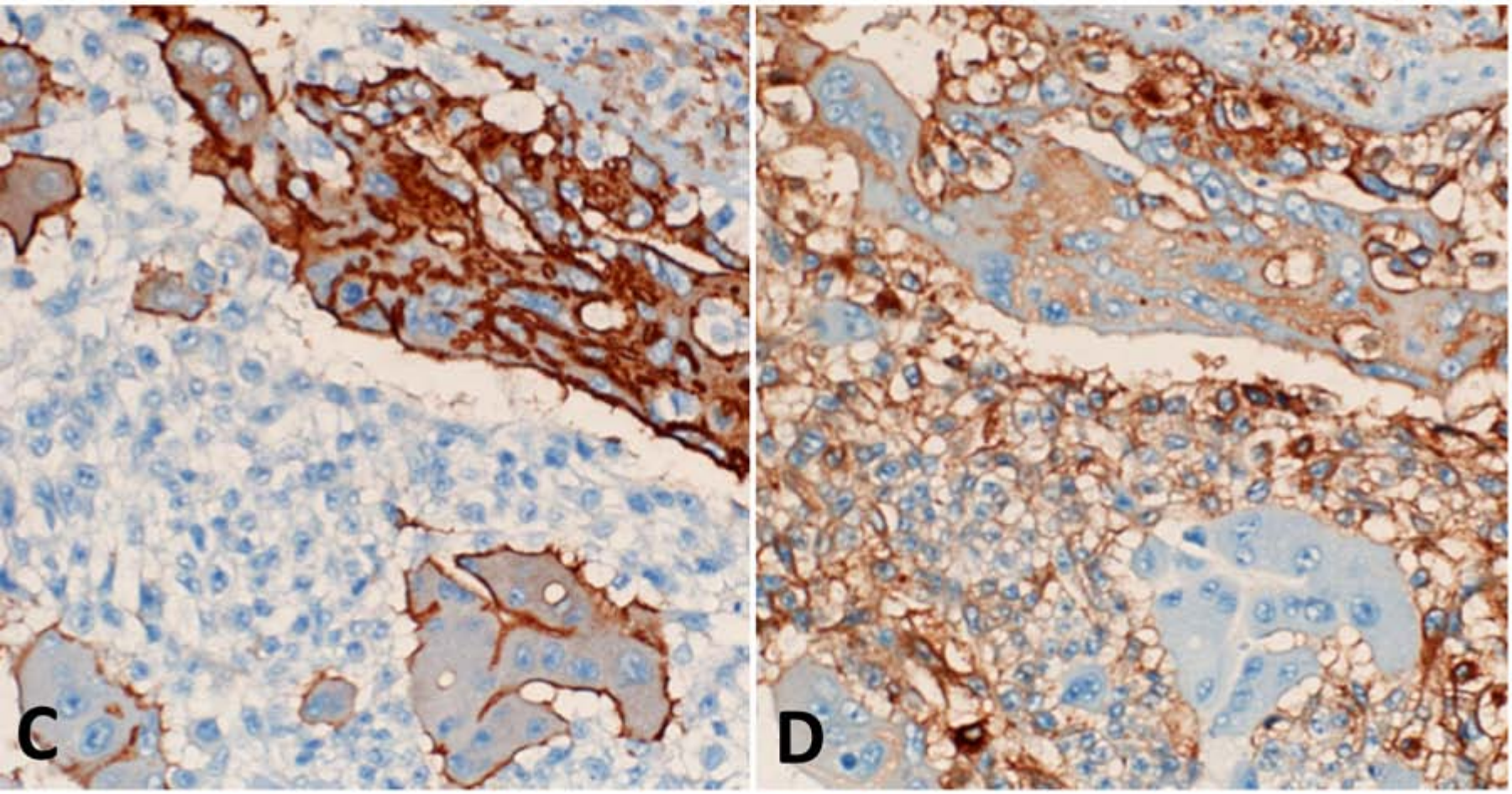
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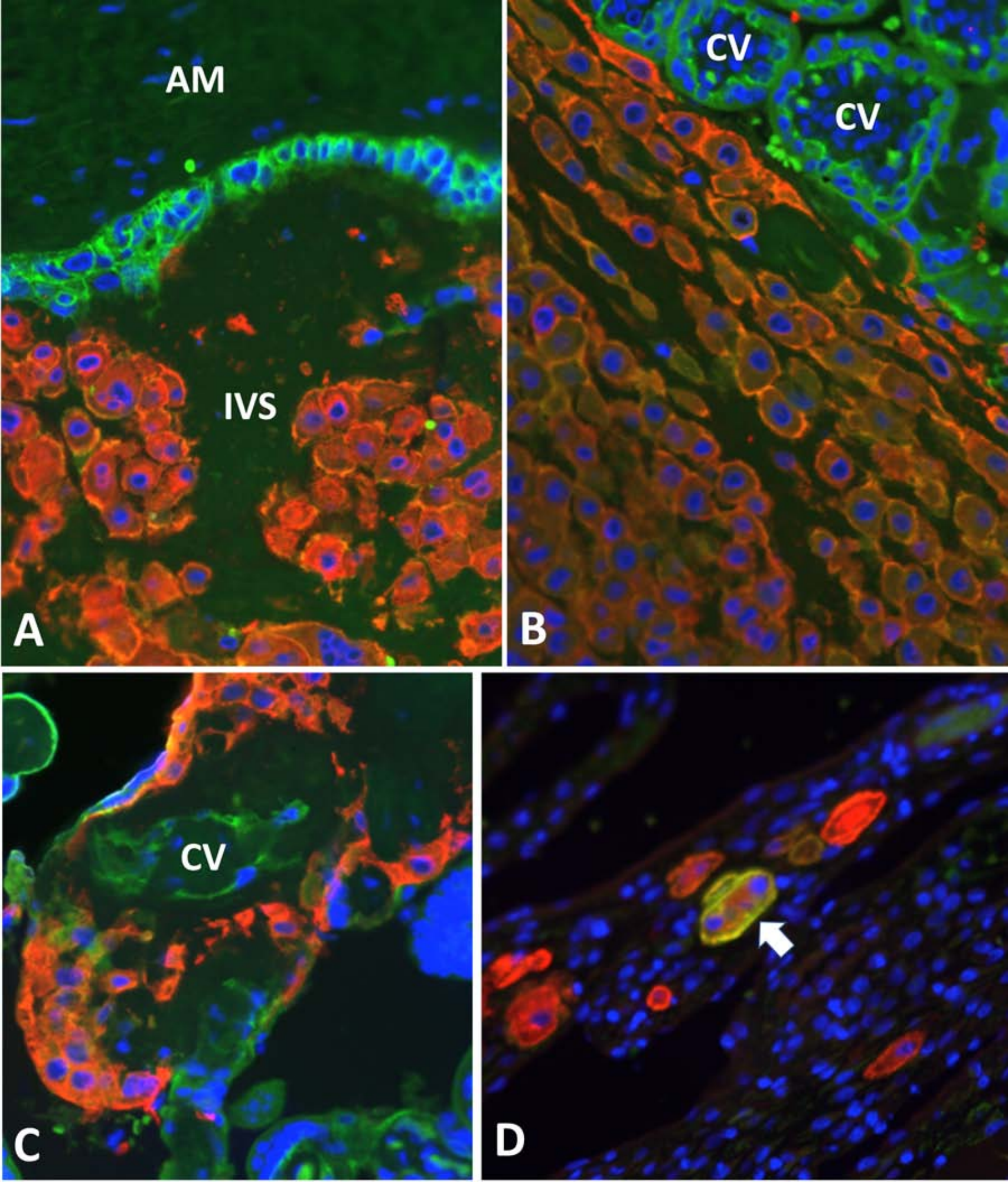
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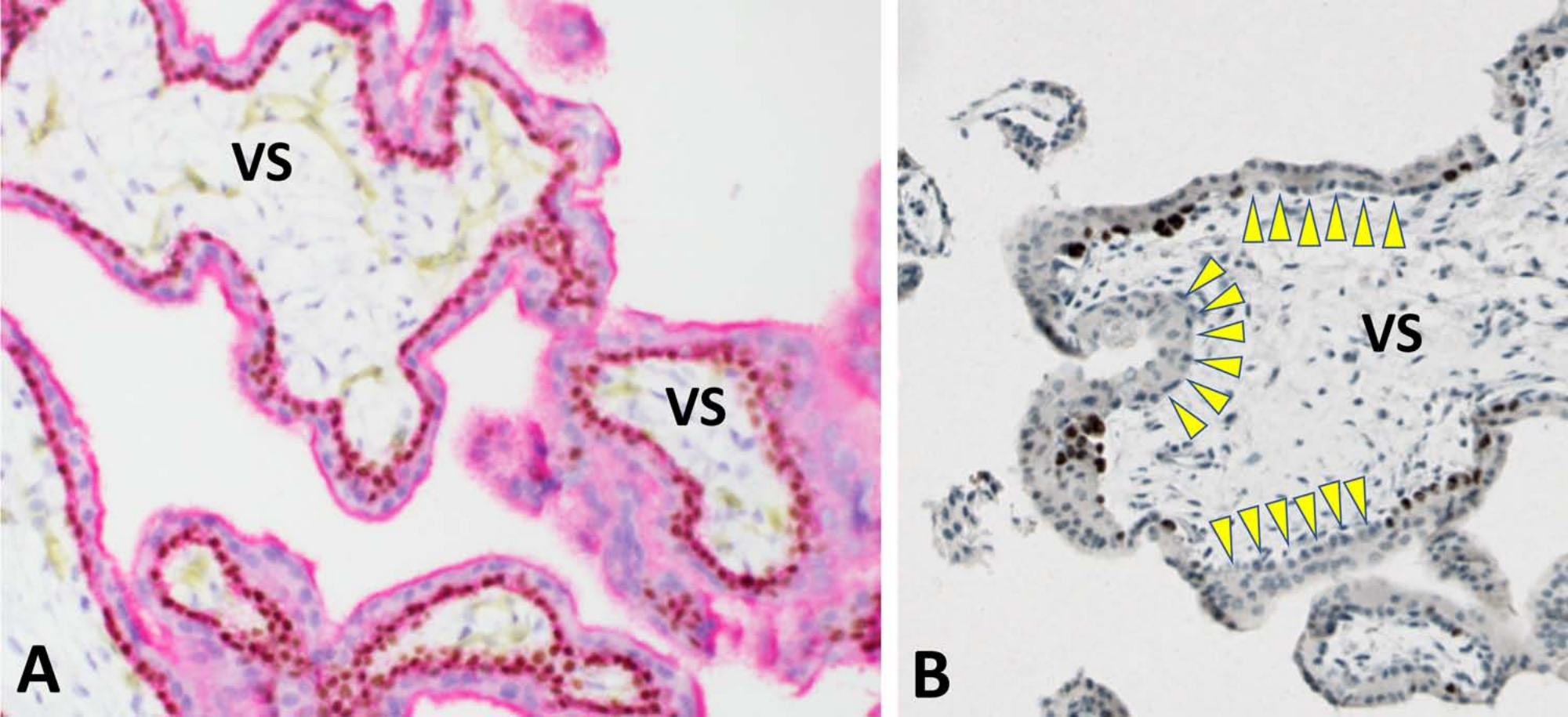
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